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Role of Methionine Sulfoxide Reductases A and B of *Enterococcus faecalis* in Oxidative Stress and Virulence[▽]

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Methionine sulfoxide reductases A and B are antioxidant repair enzymes that reduce the *S*- and *R*-diastereomers of methionine sulfoxides back to methionine, respectively. *Enterococcus faecalis*, an important nosocomial pathogen, has one *msrA* gene and one *msrB* gene situated in different parts of the chromosome. Promoters have been mapped and mutants have been constructed in two *E. faecalis* strains (strains JH2-2 and V583) and characterized. For both backgrounds, the mutants are more sensitive than the wild-type parents to exposure to H_2O_2, and in combination the mutations seem to be additive. The virulence of the mutants has been analyzed in four different models. Survival of the mutants inside mouse peritoneal macrophages stimulated with recombinant gamma interferon plus lipopolysaccharide but not in naïve phagocytes is significantly affected. The *msrA* mutant is attenuated in the *Galleria mellonella* insect model. Deficiency in either Msr enzyme reduced the level of virulence in a systemic and urinary tract infection model. Virulence was reconstituted in the complemented strains. The combined results show that Msr repair enzymes are important for the oxidative stress response, macrophage survival, and persistent infection with *E. faecalis*.

Enterococci are rather harmless commensals of the human biliary and gastrointestinal tract which belong to the group of lactic acid bacteria. Even probiotic effects are claimed for some strains. On the other hand, enterococci have emerged as important nosocomial pathogens causing wound, bloodstream, and urinary tract infections. Due to their general robustness and intrinsic and acquired resistance to antibiotics, enterococci are well equipped to survive and colonize hospital environments. Upon room colonization, patients with low levels of immunity may become colonized by these hospital strains, and it is likely that blood infections are the result of dissemination from the intestine (10).

Concerning enterococcal virulence, a few virulence factors have been characterized (see reference 13 and references therein). Esp, Ace, and pilus-like structures are thought to mediate attachment to host tissues. Aggregation substance promotes aggregation between cells, and altogether, this leads to the formation of a biofilm. Quorum sensing-based mechanisms then lead to expression of the metalloprotease gelatinase and the hemolytic cytolysin, provoking cell lysis and the spread of infection. However, the importance of these virulence factors does not always seem to be supported by the findings of clinical studies, demonstrating that we still have a lack of knowledge concerning the virulence of enterococci.

Phagocytic cells are among the first line of defense against invading pathogens. After phagocytosis, the phagocytes initiate a high-output production of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) with the aim to destroy the pathogens. The primary target is thought to be DNA, but ROIs and RNIs also react with other macromolecules, including proteins. Pathogens respond to this attack by synthesizing molecules with antioxidant activities, such as superoxide dismutases, catalases, and peroxidases, in order to neutralize these radicals, but they have also evolved systems which repair oxidative damage. The methionine (Met) residues of proteins are particularly vulnerable to oxidation, forming methionine sulfoxide (MetSO). The oxidation of Met residues actually leads to the formation of equimolar amounts of two epimers of MetSO, Met-*S*-SO and Met-*R*-SO (reaction 1): methionine + ROI (RNI) → Met-*S*-SO + Met-*R*-SO.

These MetSO residues can be reduced by methionine sulfoxide reductases (Msr), evolutionarily highly conserved enzymes able to reduce MetSO back to Met using electrons derived from thioredoxin, thioredoxin reductase, and NADPH (6, 7). Two nonhomologous Msr enzymes, named MsrA and MsrB, have been identified, and as has been shown with Msr proteins of different Gram-positive and Gram-negative bacteria, the two diastereoisomers are specifically reduced by MsrA and MsrB enzymes, according to reactions 2A and 2B (6, 20): Met-*S*-SO + MsrA → methionine (reaction 2A) and Met-*R*-SO + MsrA → methionine (reaction 2B).

The chemical mechanism involved in the reduction of MetSO to Met catalyzed by the Msr enzymes is the same and can be divided into three steps: (i) formation of a sulfenic acid intermediate on a catalytic cysteine (Cys) residue of the enzymes, leading to the release of methionine, (ii) formation of an intramonomeric disulfide bond between the catalytic Cys and a recycling Cys, and (iii) reduction of the resulting disulfide bond by the NADPH/thioredoxin reductase/thioredoxin system (6). Since *msrA* and *msrB* rank among the best-con-

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TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. faecalis strains		
JH2-2	Fus^r Rif^r, plasmid-free wild-type strain	38
JHD*msrA*	JH2-2 isogenic *msrA* deletion mutant	This study
JHD*msrB*	JH2-2 isogenic *msrB* deletion mutant	This study
JHD*msrA*/D*msrB*	JH2-2 isogenic double mutant with deletions in the aforementioned genes	This study
JH*msrA* complemented strain	Δ*msrA* strain complemented by allelic exchange	This study
JH*msrB* complemented strain	Δ*msrB* strain complemented by allelic exchange	This study
V19	Derivative of *E. faecalis* strain V583 (27) cured of its plasmids, Van^r	This study
V19*msrA*::Tet	V19 with an integration of plasmid pUCB30 in the *msrA* gene	This study
V19*msrB*::Tet	V19 with an integration of plasmid pUCB30 in the *msrB* gene	This study
E. coli Top10F′	F′ [*lacI*^{q} Tn*1*(TetR)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 araD139 galU galK* Δ(*ara-leu*)*7697 rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pUCB30	oripMB1 *lacZ′* Amp^r Em^r	5
pMAD	oripE194^{ts} Em^r Amp^r *bgaB*	3
pVE14218	oripWV01 RepA$^-$ Tet^r	L. Rigottier-Gois and P. Serror, unpublished data
pGhost3	oripWV01 RepA^{ts} Cm^r	21

served genes in nature, it is suggested that their products have very important functions for cellular life, most impressively demonstrated by the finding that Msr deficiency affects longevity in yeast and mammals (15, 22, 23, 25). With one exception (39), the bacterial *msrA* mutants tested were more sensitive to exposure to oxidants, such as H_2O_2, organic hydroperoxides, and/or nitric oxide (26, 30). Hitherto, only a few studies have addressed the role of MsrB in bacterial resistance to oxidative stress. While a *Helicobacter pylori msrB* mutant was more sensitive to three different oxidants, this mutation had no effect on oxidative stress resistance in *Mycobacterium tuberculosis* (1, 20). Besides that, the copy number and genetic organization of *msr* genes vary widely among the different organisms. The majority of microorganisms contain one copy of each gene in two different transcription units (12, 30). In other bacteria, the *msrA* and *msrB* genes are part of the same operon, and the two genes are sometimes translationally fused. Some bacteria have more than one *msrA* and/or *msrB* paralog, which are most frequently present on the chromosome but which are also sometimes present on a plasmid (12, 30).

Convincing data indicating a close relationship between Msr and bacterial virulence has accumulated in recent years. For different pathogenic bacteria, the reduced virulence of *msr*-deficient strains as well as effects on bacterial adherence, biofilm formation, intracellular survival, and colonization capabilities has been reported (4, 9, 37). In the present work we address the question of whether the *msrA* and *msrB* genes of *Enterococcus faecalis* are important in the oxidative stress response and virulence of this opportunistic pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. Cultures of *E. faecalis* strains were performed on M17 medium (33) supplemented with 0.5% (wt/vol) glucose (GM17). Overnight cultures of the *E. faecalis* strains were grown at 37°C without shaking in 30-ml glass tubes containing 10 ml GM17. When appropriate, erythromycin (150 μg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 80 μg ml^{-1}) were added to the medium. *Escherichia coli* strains were cultivated under vigorous agitation at 37°C in Luria-Bertani (LB) medium (29) with ampicillin, when it was required. Growth was followed by measuring the optical density at 600 nm (OD_{600}) on a Biophotometer (Eppendorf).

Survival experiments. Overnight cultures of *E. faecalis* strains were prepared. The cells were harvested by centrifugation of 2 ml of the overnight culture. The cells were resuspended in 10 ml of M17 medium. A 96-well microtiter plate was prepared as follows: lane 1 to lane 8 contained 20 μl of 0 mM, 5 mM, 6 mM, 7 mM, 7 mM, 6 mM, 5 mM, and 0 mM H_2O_2, respectively. A total of 180 μl of the resuspended cultures was added to each well using an eight-channel pipette (Eppendorf Research Plus). This setup allows testing of 12 strains per microtiter plate. The plates were incubated for 2 h at 37°C without agitation. Then, 20 μl was recovered and used to inoculate another plate containing 180 μl of GM17 in each well. The plates were put into a microtiter plate reader (model 680; Bio-Rad). Before reading of the OD_{600}, the plates were agitated for 10 s. The incubation temperature was 37°C, and readings were performed every 30 min for 24 h.

Construction of insertion and unmarked deletion mutants and of complemented strains. Primers were purchased from Operon (Cologne, Germany). The general protocol for the construction of deletion mutants using plasmids pUCB30 and pMAD is detailed elsewhere (16). Briefly, a DNA chromosomal fragment of 3.5 kbp comprising the *msrA* or *msrB* gene to be deleted was amplified by PCR using *Pfu* ultraproofreading DNA polymerase (Stratagene) and corresponding oligonucleotides deduced from the *E. faecalis* V583 sequence (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=gef) with overhanging 5′ ends containing recognition sites for restriction endonucleases (REs). Each PCR fragment was then purified with a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany), cut with the corresponding RE, and ligated into cloning vector pUCB30 and, for the complementation experiments, cloning vector pMAD (see below). Recombinant plasmids were purified, diluted (1 ng/μl), and used as templates for inverse PCR with *Pfu* ultrapolymerase in order to introduce the deletion. This introduced central deletions of 300 bp and 328 bp in the *msrA* and *msrB* genes, respectively. After cloning of the sequences into the pMAD vector (3), *msrA* and *msrB* deletion mutants were obtained following the procedures described previously (16). The single Δ*msrA* mutant was subsequently used to construct the double Δ*msrA* Δ*msrB* mutant using the same protocol. All mutants were finally verified to have deletions of the copies of the *msrA* and *msrB* genes as well as to lack plasmid pMAD by PCR analysis. The

complementation of each single mutant was performed by knocking in the wild-type allele into the corresponding mutant using pMAD, as described previously (36).

Single-crossing-over insertion-duplication mutagenesis was based on a two-vector system and was performed essentially as described previously (18) using plasmid pG$^+$host (RepA^{ts} [temperature sensitive {ts}]) and integrative plasmid pVE14218 (Tet^r).

Animal studies. *Galleria mellonella* larvae were reared on beeswax and pollen at 37°C in darkness. The *E. faecalis* strains used for infection were grown for 24 h in GM17. After centrifugation of 4 ml of each culture, cells were washed twice in 1 ml of 0.9% NaCl. The bacterial cells were then resuspended in 0.9% NaCl to get a suspension with an OD_{600} of 1.2. For each strain, 15 *G. mellonella* caterpillars of about the same size (body weight, 200 to 300 mg) were infected, and each experiment was repeated at least three times. Ten microliters of the cell suspension was injected into the second hindmost proleg of each *G. mellonella* larva using an automatic syringe pump (KD Scientific, Holliston, MA). After injection, the caterpillars were incubated at 37°C in petri dishes, and the number of survivor caterpillars was scored every 2 h after 15 h of infection. Caterpillars were considered dead when they displayed no movement in response to touch and had turned black.

Mouse experiments were performed with the approval of an institutional animal use committee. Female BALB/c mice (weight, 20 to 25 g; Harlan Italy S.r.l) were housed in filter-top cages at the Catholic University Unit for Laboratory Animal Medicine and had free access to food and water. Two peritoneal macrophage models were used to test the survival of *E. faecalis* single and double mutants and of the complemented mutants. The first is a previously described *in vivo*/*in vitro* infection model (14). In the second model, peritoneal macrophages were isolated from mice 4 days after intraperitoneal injection of 2 ml of a sterile 10% thioglycolate solution and cultured as described previously (8, 31). Macrophages were plated in 24-well (2×10^6 cells/well) tissue culture plates (Corning). After overnight incubation, the cells were washed with phosphate-buffered saline (PBS) to remove nonadherent cells and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 mM HEPES, 2 mM glutamine, 10% bovine fetal serum, and 1× nonessential amino acids (DMEM complete medium) for 24 h before treatment. The macrophages (>99%, as evaluated by anti-mouse F4/80 antibody staining) were stimulated with medium alone or recombinant gamma interferon (rIFN-γ; 20 ng/ml; Sigma-Aldrich, Milan, Italy) plus lipopolysaccharide (LPS; 10 ng/ml; Sigma-Aldrich) for 24 h and then infected with *E. faecalis* cells (multiplicity of infection = 10) in DMEM complete medium and incubated for 2 h at 37°C to permit phagocytosis of the *E. faecalis* cells. The macrophages were washed with PBS and placed in DMEM complete medium with vancomycin (10 μg/ml) and gentamicin (10 μg/ml) to kill extracellular bacteria. To quantify the number of intracellular *E. faecalis* cells, the macrophages were washed and then lysed with detergent at different time points (2, 5, 8, 24, 48, and 72 h). After dilution with PBS, the lysates were plated onto *Enterococcus* selective agar (ESA; Fluka Analytical, Switzerland) to determine the numbers of CFU of viable intracellular bacteria. All experiments were performed at least three times, and the results were analyzed by one-way analysis of variance (ANOVA) with a Bonferroni correction posttest.

In addition, to assess the virulence of the *E. faecalis* single and double mutants and the complemented mutants for the JH2-2 wild-type strain, two mouse models were used. In the intravenous infection model, experiments were performed as described previously (14). Briefly, overnight cultures of the strains grown in brain heart infusion (BHI) broth supplemented with 40% heat-inactivated horse serum were centrifuged, and the resulting pellets were resuspended in sterile PBS to achieve final concentrations of 1×10^9 bacteria/ml. Aliquots of 100 μl from each strain suspension were used to inject the tail veins of groups of 10 mice each. The infection experiments were repeated three times. The mice were monitored with twice-daily inspections, and at 7 days after infection they were killed using CO_2 inhalation. The kidneys and livers were then removed aseptically, weighed, and homogenized in 5 ml of PBS for 120 s at high speed in a Stomacher 80 apparatus (Pbi International, Milan, Italy). Serial homogenate dilutions were plated onto ESA medium for determination of the numbers of CFU.

In the urinary tract infection (UTI) model, we followed a previously described protocol (19). Briefly, each bacterial strain was grown in 10 ml of BHI broth supplemented with 40% heat-inactivated horse serum for 10 h at 37°C with shaking. The cells were pelleted, resuspended in 10 ml of sterile PBS, and adjusted to reach a concentration of 1×10^7 bacteria/ml. Groups of five isoflurane-anesthetized mice per bacterial inoculum (10^2 to 10^6 CFU) were first infected with 200 μl of each strain suspension via intraurethral catheterization (polyethylene catheter ~4 cm long; outer diameter, 0.61 mm; Becton Dickinson, Sparks, MD). Additionally, groups of 15 mice each were infected with a sole inoculum of 10^4 CFU. The mice were killed 48 h after transurethral challenge, and the bladders and kidney pairs were processed as described above. For each strain, the 50% infective dose (ID_{50}) was determined as described previously (28). The bacterial detection limits were 50 and 10 CFU/ml for the kidney and bladder homogenates, respectively. Differences between the total numbers of infected kidney pairs or bladders, obtained by combining the data for all inoculum (10^2 to 10^6 CFU) groups, were analyzed by Fisher's exact test. For both models, CFU counts were analyzed by the unpaired *t* test. All statistical analyses were performed using Prism software (version 5.00) for Windows (GraphPad Software, San Diego, CA). For all comparisons, a *P* value of less than 0.05 was considered significant.

RESULTS

Definition of *msrA* and *msrB* operon structures and promoter localizations. Analysis of the genome sequences of different *E. faecalis* strains available at The Institute for Genome Research (TIGR; strain V583) and at the Human Genome Sequencing Center at Baylor College of Medicine (strains OG1RF, TX0104, and HH22) revealed that all strains harbor one *msrA* gene and one *msrB* gene, located on different parts of the chromosome. Sequence analysis of the MsrA and MsrB proteins of *E. faecalis* revealed that they show significant identity with several prokaryotic Msr proteins (data not shown) and that they are part of the third and first subclasses of MsrA and MsrB enzymes, respectively (6). The third class of MsrA enzymes, represented by the *Bacillus subtilis* protein, has an N-terminal CFWC thioredoxin-like signature, also present in the enterococcal homologue, which is supposed to correspond to the catalytic center of the enzyme (24). The first subclass of MsrB enzymes is represented by the *Neisseria meningitidis* protein, in which C-117 (C-118 in *E. faecalis*) corresponds to the catalytic Cys and C-63 (the position is conserved in the *E. faecalis* enzyme) corresponds to the regeneration Cys (6).

The genetic context of each *msr* gene of *E. faecalis* strain V583 is shown in Fig. 1 and 2; this organization is also conserved in *E. faecalis* strains OG1RF, TX0104, and HH22. The *E. faecalis msrA* gene (*ef1681*) is in tandem organization with three other genes (*ef1683*, *ef1682*, and *ef1680*) transcribed counterclockwise and before the last gene in this structure. The four genes are separated by short intergenic regions (1 bp, 16 bp, and 7 bp) and are flanked by putative transcription terminators (Fig. 1A). These characteristics suggested that the four genes form an operon, named the *msrA* operon throughout this article. The *ef1683* gene encodes a putative lipase/acylhydrolase of unknown function, and the *ef1682* and *ef1680* genes encode conserved hypothetical proteins. In order to experimentally verify the presumed operon structure, RT-PCR experiments were performed (Fig. 1B). This confirmed that the *msrA* gene is cotranscribed with *ef1683*, *ef1682*, and *ef1680* but also revealed the presence of transcripts containing sequences upstream of *ef1683* and the putative transcription terminator present in the *ef1684-ef1683* intergenic region. This suggested the existence of multiple promoters, with one also mapping upstream of the stem-and-loop structure. The promoters were mapped by 5′ rapid amplification of cDNA ends-PCR (RACE-PCR), and the oligonucleotides used were designed to be able to identify transcriptional start sites situated downstream as well as upstream of the putative terminator. These experiments showed that the only promoter mapped, named P_{msrA} (Fig. 1A and D), was situated upstream of the transcriptional terminator in the 3′ end of the *ef1684* gene, leading to the synthesis of

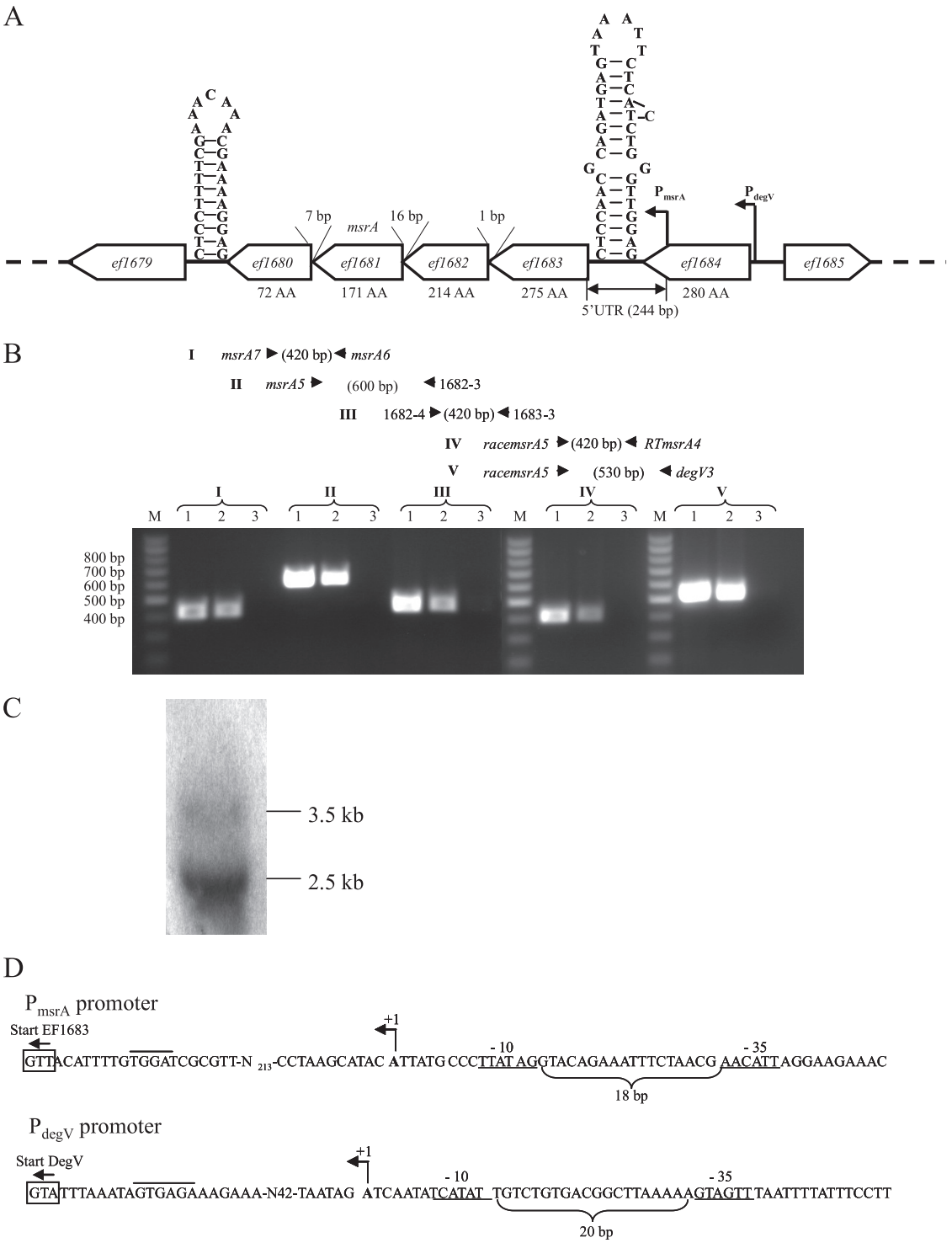

FIG. 1. (A) Genetic context of the *msrA* gene of *E. faecalis*. The open reading frames are represented by open arrows, and their orientation indicates the transcriptional direction. Numbers above the intergenic regions indicate the gene distances. The numbers below the genes indicate the sizes of the gene products in amino acids (AA). The size of the 5′ UTR is given in bp. The nucleotide sequences of the putative *rho*-independent terminators, located 4 and 8 nucleotides downstream of the *ef1680* and *ef1684* stop codons, respectively, are shown. P_{msrA} and P_{degV} indicate the positions of two promoters mapped by 5′ RACE-PCR (see panel D) (B) Positions of hybridization of 5 oligonucleotide pairs (I to V) used for RT-PCR. The expected amplimer sizes are indicated in bp. The results of the RT-PCRs with the five primer pairs are shown in the electropherogram. Lanes 1, control PCR with chromosomal DNA; lanes 2, RT-PCR; lanes 3, PCR with RNA extraction without prior reverse transcriptase reaction (negative control); lanes M, molecular size standard, with sizes given at the left of the gel. (C) Northern blot analysis using RNA extracted from exponentially growing cells. Hybridization was performed with an [α-^{32}P]dATP-labeled single-strand probe complementary to the *msrA* mRNA. The size of the transcript was estimated by comparison with the sizes on an RNA ladder. (D) Mapping of promoters P_{msrA} and P_{degV} by 5′ RACE-PCR. The transcriptional initiation sites (+1) are indicated, the putative −35 and −10 motifs are underlined, and the distances between the two boxes are given in bp. The putative ribosome binding sites are overlined, and the start codons are boxed.

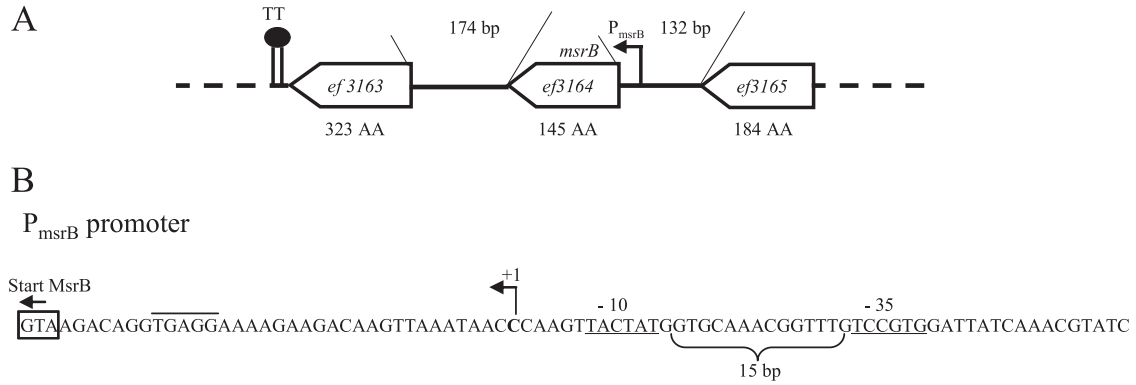

FIG. 2. (A) Genetic context of the *msrB* gene of *E. faecalis*. For detailed information, see the legend for Fig. 1A. TT, position of a putative *rho*-independent transcriptional terminator located 8 nucleotides downstream of the *ef3163* stop codon. (B) The transcription start site of the *msrB* operon has been mapped using 5′ RACE-PCR (for details, refer to the legend for Fig. 1D).

a transcript with a 244-bp untranslated region (UTR). P_{msrA} displays −10 (GATATT) and −35 (TTACAA) regions separated by 18 bp whose sequences differ from the consensus sequences (TATAAT and TTGACA) of the bacterial promoters at two and three positions, respectively. However, in some electropherograms the sequence seemed to continue after the poly(G) tail added at the 5′ ends during the RACE-PCR protocol, which suggested the existence of a longer transcript as well. Another RT-PCR experiment using oligonucleotides *racemsrA5* and *degV3* (mapping upstream of P_{msrA}) indeed revealed a PCR product, strengthening our hypothesis (Fig. 1B). For mapping the second supposed promoter for expression of the *msrA* operon, another round of 5′ RACE-PCR was conducted. The only promoter which was identified is situated upstream of the *ef1684* gene. This last gene encodes a putative DegV family protein of unknown function, and the promoter identified was named P_{degV} (Fig. 1D). This promoter has characteristics similar to those of the consensus sequences in the −10 element (TATACT) and −35 element (TTGATG), which are separated by 20 bp. However, as judged by Northern blot analysis (Fig. 1C), the main promoter for the expression of the *msrA* operon seems to be P_{msrA}, since the main transcript of approximately 2.5 kbp is compatible if the *msrA* operon is expressed from this *degV* internal promoter. Nevertheless, a minor transcript with a size of approximately 3.5 kbp also seems to exist on the Northern blot and may correspond to a pentacistronic transcript containing the *msrA* operon and *degV* expressed from P_{degV}.

The *E. faecalis msrB* gene (*ef3164*), annotated as a PilB family protein in the TIGR database, was already the subject of a previous work (17). In that study, it has been shown that *msrB* expression is induced by heavy metals, and it was suggested from the results of Northern blot experiments using total RNA preparations from cadmium-induced cultures that *msrB* forms an operon with the downstream end of the *ef3163* gene encoding a ribose-phosphate pyrophosphokinase implicated in purine ribonucleotide biosynthesis. Both genes are transcribed counterclockwise and are separated by a relatively long intergenic region of 174 bp in which no putative transcription terminator could be detected (Fig. 2A). In the present work, we mapped the transcriptional start site by 5′ RACE-PCR, which showed that it is located 34 bp upstream of the ATG translational start codon of the *msrB* gene. Upstream of this point, regions similar to consensus −10 and −35 boxes separated by 15 bp are found (Fig. 2B). This experimentally defined promoter is different from that proposed in the previous study (17).

Effect of *msrA* and *msrB* gene inactivation on *E. faecalis* resistance to oxidative stress. In order to define the physiological roles of the *E. faecalis* Msr proteins, mutants with markerless single and double deletions have been constructed in strain JH2-2. Furthermore, insertional *msr* mutants have been

TABLE 2. Sensitivities of Msr-deficient isolates of two *E. faecalis* strains toward exposure to three different H_2O_2 concentrations

Strain	Time (h) to reach OD of 0.2 with H_2O_2 concn of[a]:			
	0 mM	5 mM	6 mM	7 mM
JH2-2	0.6 ± 0.1	5.2 ± 0.8	6.4 ± 0.8	8.2 ± 0.8
JHΔ*msrA*	0.6 ± 0.1	7.3 ± 0.9 ($P = 0.037$)	8.2 ± 0.8 ($P = 0.067$)	10.0 ± 0.8 ($P = 0.18$)
JH*msrA*comp[b]	0.6 ± 0.1	5.9 ± 0.7	7.0 ± 0.6	8.5 ± 0.9
JHΔ*msrB*	0.6 ± 0.1	10.0 ± 0.5 ($P = 0.001$)	12.9 ± 0.9 ($P = 0.001$)	15.6 ± 1.4 ($P = 0.002$)
JH*msrB*comp[b]	0.6 ± 0.1	5.0 ± 0.5	6.2 ± 0.5	7.9 ± 0.9
JHΔ*msrA/B*	0.6 ± 0.1	12.2 ± 0.6 ($P < 0.001$)	14.2 ± 1.5 ($P = 0.002$)	17.6 ± 1.5 ($P = 0.001$)
V19[c]	1.4 ± 0.1	7.4 ± 0.7	8.3 ± 1.0	9.4 ± 1.6
V19*msrA*::Tet	1.5 ± 0.1	12.7 ± 2.0 ($P = 0.001$)	14.5 ± 2.5 ($P = 0.002$)	16.5 ± 2.5 ($P = 0.002$)
V19*msrB*::Tet	1.4 ± 0.15	13.3 ± 1.3 ($P < 0.001$)	14.3 ± 1.0 ($P < 0.001$)	15.7 ± 1.6 ($P = 0.001$)

[a] The results are expressed as the means of three independent experiments. Standard deviations are indicated.
[b] JH*msrA* and JH*msrB*comp correspond to the Δ*msrA* and Δ*msrB* complemented strains, respectively.
[c] Strain V19 is a derivative of strain V583 cured of its plasmids.

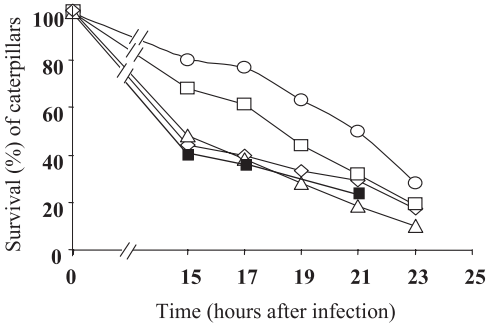

FIG. 3. Virulence test of *msr* mutants using the *Galleria mellonella* insect model. About 5×10^8 CFU was injected into 1 caterpillar, and 15 caterpillars were used for each strain. Viable caterpillars infected with the JH2-2 wild-type strain (◇), the Δ*msrA* mutant (□), the Δ*msrB* mutant (△), the Δ*msrA* Δ*msrB* double mutant (○), and the Δ*msrA* (■) complemented strain were counted after 15, 17, 19, 21, and 23 h after infection.

constructed in a plasmid-cured strain (V19) of *E. faecalis* V583. The growth of all mutant strains either with or without agitation or in the presence of different concentrations of H_2O_2 (1.5 mM, 2 mM, 2.5 mM, and 3 mM) was comparable to that of the corresponding wild-type strains (data not shown). The *msr* mutants were then tested for resistance to H_2O_2 (Table 2). In this test system, cells are exposed to a given H_2O_2 concentration for 2 h. After the treatment, an aliquot is used to inoculate fresh medium and the time needed by the culture to reach an OD of 0.2 is determined. This showed that in both *E. faecalis* strains, the time of outgrowth of the *msr* mutants was always longer than that for their wild-type counterparts, indicating that they are more sensitive to the peroxide treatments. The Δ*msrA* Δ*msrB* double mutant constructed in strain JH2-2 is more sensitive than the single mutants, suggesting an additive effect of the mutations. Comparing the results between both strains showed that in the JH2-2 background the *msrA* mutant was less affected than the *msrB* mutant, whereas the corresponding *msr* mutants constructed in the plasmid-cured derivative of strain V583 demonstrated comparable sensitivities.

Effects of *msrA* and *msrB* gene deletion on *E. faecalis* virulence. In order to assess the effects of the Msr enzymes on the virulence of *E. faecalis*, different animal models were used. To exclude the stability problems frequently observed with insertional mutants, only the stable deletion mutants constructed in strain JH2-2 were tested. Moreover, complemented strains were constructed for the Δ*msrA* and Δ*msrB* mutants. We started our analysis on the impact of Msr deficiencies on virulence in the simple *Galleria mellonella* insect model (Fig. 3), which has proved useful for the screening of virulence factors of pathogens (13). The level of killing of the insect larvae by the Δ*msrB* mutant was comparable to that of the wild-type control. The virulence of the Δ*msrA* mutant seems attenuated in this model, at least at 15 and 17 h after infection ($P = 0.045$). However, the combination of both mutations in one strain led to the most significant attenuation of virulence in this model ($P = 0.039$). The survival capacities of the different strains inside mouse peritoneal macrophages were then measured using an *in* vivo/*in vitro* model described previously (16). All

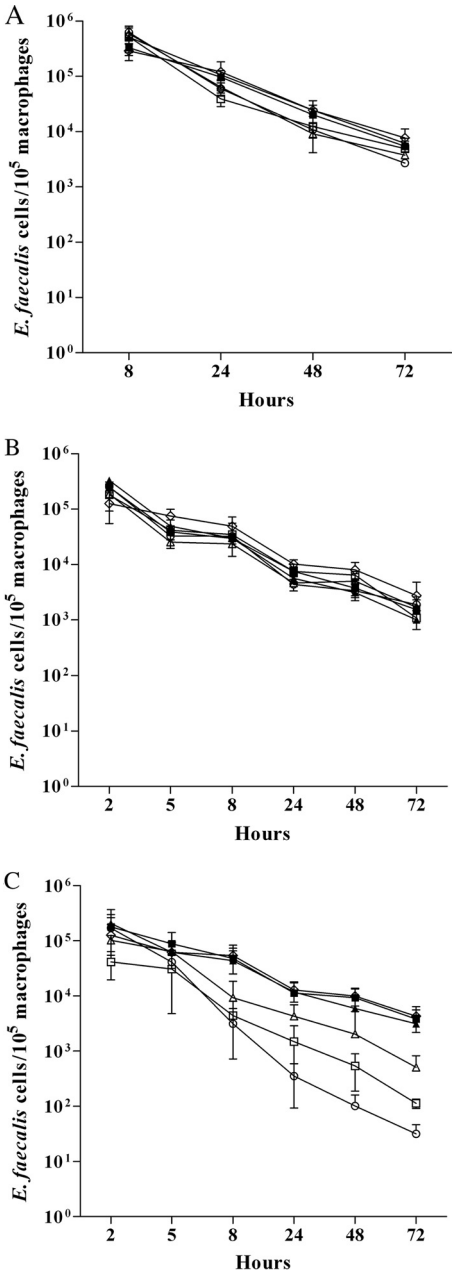

FIG. 4. Survival of the *E. faecalis* JH2-2 wild type (◇); its isogenic Δ*msrA* (□), Δ*msrB* (△), and Δ*msrA* Δ*msrB* (○) mutant strains; and the Δ*msrA* (■) and Δ*msrB* (▲) complemented strains in mouse peritoneal macrophages derived from an *in vitro*/*in vivo* infection model (A) or isolated from the animals and cultured *in vitro* with medium alone (B) or with rIFN-γ plus LPS (C) before infection. The data are expressed as means ± standard deviations for the number of viable intracellular bacteria per 10^5 macrophages in at least three different experiments.

strains survived equally well in this test system (Fig. 4A). While the same results were also obtained using isolated mouse peritoneal macrophages without stimulation (Fig. 4B), differences were noted when the macrophages were stimulated by rIFN-γ plus LPS before infection. Under these conditions, the rates of survival of the Δ*msrA*, Δ*msrB*, and Δ*msrA* Δ*msrB* mutants were

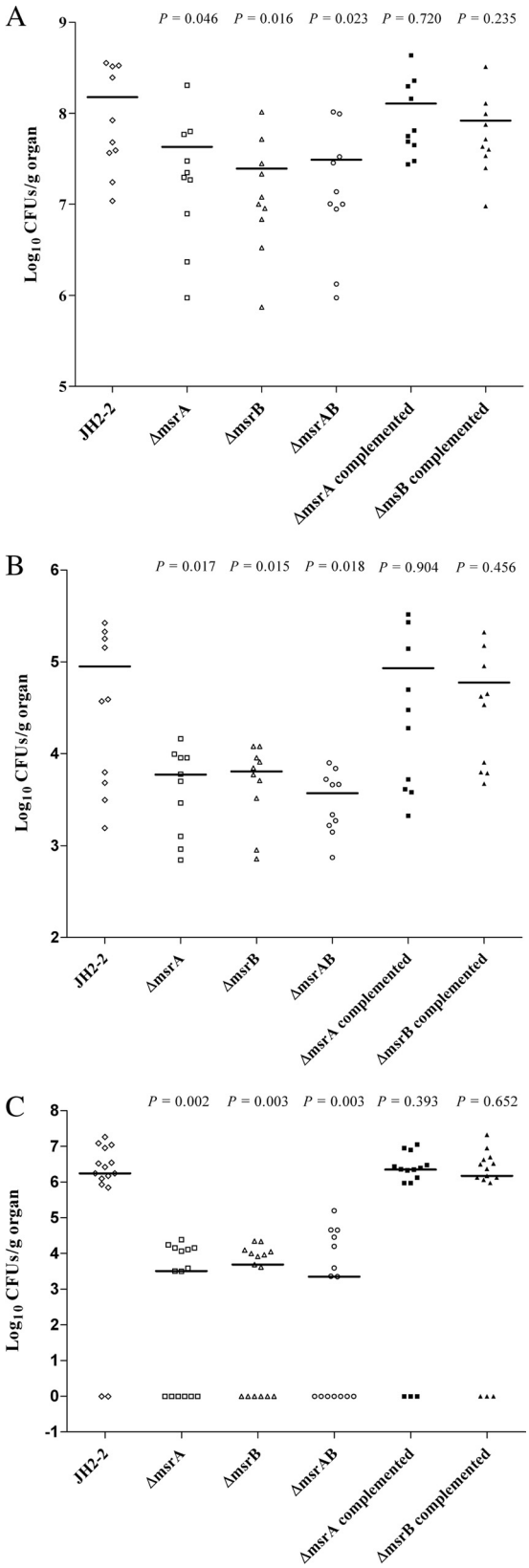

FIG. 5. Enterococcal tissue burdens in kidneys (A) and livers (B) of BALB/c mice infected intravenously with 5×10^8 cells of the *E. faecalis* JH2-2 wild type (◇); its isogenic Δ*msrA* (□), Δ*msrB* (△), and Δ*msrA* Δ*msrB* (○) mutant strains; and the Δ*msrA* (■) and Δ*msrB* (▲) complemented strains. Groups of 10 mice were killed and necropsied at day 7 postinfection. (C) Enterococcal burdens of the kidneys of BALB/c mice infected transurethrally with 10^4 cells of the *E. faecalis* JH2-2 wild type (◇); its isogenic Δ*msrA* (□), Δ*msrB* (△), and Δ*msrAB* (○) mutant strains; and the *msrA* (■) and *msrB* (▲) complemented strains. Kidney pair homogenates were obtained from groups of 15 mice that were killed and necropsied 48 h after the transurethral challenge. The results, expressed as $\log_{10}$ CFU per gram of tissue, represent the values recorded separately for each mouse. Horizontal bars represent the geometric means. A value of 0 was assigned to uninfected kidneys.

significantly lower than those of the wild-type strain at 8 h ($P = 0.002$, $P = 0.0049$, and $P = 0.0016$, respectively), 24 h ($P = 0.0011$, $P = 0.0019$, and $P = 0.0001$, respectively), 48 h ($P < 0.0001$, $P = 0.0014$, and $P < 0.0001$, respectively), and 72 h ($P < 0.001$, $P = 0.0011$, and $P < 0.0001$, respectively) (Fig. 5C). We next compared the burdens in the kidney and liver tissues of groups of infected mice using a well-established intravenous infection model (14). As shown in Fig. 5, all mutants (Δ*msrA*, Δ*msrB*, and the Δ*msrA* Δ*msrB* double mutant) showed statistically significant reductions in the burdens in both kidney and liver tissues. In detail, the Δ*msrA* mutant exhibited reductions of 0.54 log unit in the kidneys ($P = 0.046$) and 1.18 log units in the livers ($P = 0.0179$) compared to the burdens of the JH2-2 wild-type strain and reductions similar to those obtained for the Δ*msrB* mutant (0.78 log unit in kidneys [$P = 0.016$]; 1.15 log units in livers [$P = 0.0159$]). Virulence was reconstituted to the wild-type level in the *msrA* and *msrB* complemented strains (Fig. 5A and B). The results obtained for the double mutant, showing reductions of 0.69 log unit in the kidneys ($P = 0.023$) and 1.38 log units in the livers ($P = 0.0184$), confirmed the involvement of MsrA and MsrB in *E. faecalis* virulence. Finally, the same strains were also tested in a UTI model described elsewhere (19). The resulting ID_{50}s showed that the Δ*msrA*, Δ*msrB*, and double mutant strains required 0.66, 0.68, and 1.11 $\log_{10}$ more cells (2.5×10^3, 2.6×10^3, and 7.0×10^3, respectively) than wild-type strain JH2-2 (5.4×10^2) to infect 50% of mice. In addition, for all the bacterial inocula used, the proportions of infected kidneys were 84% for JH2-2, 64% for the both Δ*msrA* and Δ*msrB* single mutant strains ($P = 0.02$), and 56% for the double mutant strain ($P = 0.002$). As a representative example, Fig. 5C shows the $\log_{10}$ numbers of CFU recovered from the kidney pairs of mice infected with 10^4 cells from JH2-2 or each of the mutant strains. As expected, all three mutant strains exhibited statistically significant reductions (2.85 log units for both Δ*msrA* and Δ*msrB* [$P = 0.002$]; 2.39 log units for the double mutant [$P = 0.003$]) in kidney tissue burden compared to the reduction for wild-type strain JH2-2. In the UTI model, the *msrA* and *msrB* complemented strains also showed a wild-type level of virulence (Fig. 5C).

DISCUSSION

E. faecalis is part of those bacteria in which the *msrA* and *msrB* genes constitute two separate transcription units located in different parts of the genome. The *msrA* gene of *E. faecalis* is embedded in a complex operon structure and seems to be expressed from two different promoters, resulting in the syn-

thesis of four-gene and five-gene polycistronic messengers. Expression from both promoters led to the synthesis of a transcript with a long 5′ UTR containing a structure resembling a transcriptional terminator. This structure might be implicated in the regulation of expression of the *msrA* operon. In some bacteria, *msrA* expression is induced by oxidants (2, 35), although in most bacteria investigated so far, this was not the case (30). In *E. coli* and *Staphylococcus aureus*, a modest induction of *msrA* in stationary phase has also been reported (26, 32). Our preliminary results obtained by reverse transcription-quantitative PCR and from microarrays using RNAs extracted from, respectively, cultures treated for 30 min with 2.5 and 1.5 mM H_2O_2 suggests that *msrA* transcription is not induced under these conditions (data not shown). However, in order to get closer insight into the regulation of this gene, more sophisticated approaches based on specific anti-MsrA antibodies or use of a reporter gene assay are necessary.

Comparison of the *msrA* genome region with the regions of other bacteria using the region comparison tool of the TIGR database revealed that the most closely related genetic context is present in *Lactobacillus casei* ATCC 334. Indeed, it has an identical succession of the five genes which are close homologues of the corresponding *E. faecalis* genes. Furthermore, as in *E. faecalis*, the *degV* gene is separated from the gene encoding the putative lipase by a long intergenic region containing a transcription terminator. The corresponding genomic region in *Listeria monocytogenes* differs from the organization in *E. faecalis* by the insertion of the *msrB* gene in conjunction with a gene encoding a putative dehydrogenase between the *msrA* gene (*ef1681*) and the last gene (*ef1680*). However, in *L. monocytogenes* the long intergenic region is absent between the *degV* gene and the following gene, and it has recently been found that the *degV* gene is an integral part of the *msrA-msrB* operon (34).

Our physiological studies showed that both the *msrA* and the *msrB* genes are important to protect cells against exposure to H_2O_2 concentrations. However, relatively high oxidant concentrations are needed to see differences that seem beyond physiological relevance. A survey of the literature indicated that most bacteria lacking MsrA investigated so far displayed sensitivity to this oxidant (30). Only a few studies concerned the role of *msrB* in the resistance to H_2O_2. Whereas the *Helicobacter pylori msrB* mutant was sensitive (1), the corresponding mutant of *Mycobacterium smegmatis* had no role in the defense to this oxidant (30).

No difference in survival inside macrophages of the *msr* mutants was evidenced using the *in vitro/in vivo* model (14). This was in apparent contrast to other findings showing that mutants deficient for well-known antioxidant activities of *E. faecalis*, like superoxide dismutase and thiol peroxidase, were hypersensitive in this test system (16, 36). However, when the *msr* mutants were tested in IFN-γ-activated macrophages, their rate of survival was lower than that of the parent strain. This was in agreement with the concept that oxidative killing is normally regulated by IFN-γ in macrophages, thus implying that the *msr* mutants displayed a phenotype only under intense oxidative stress conditions. This is consistent with the growth and survival experiments in the presence of H_2O_2. Differences were seen only when H_2O_2 concentrations of >5 mM were used. Of note, the reduction in the rate of intracellular survival of a *msrA* mutant strain of *M. smegmatis* (a *msrB* mutant was not tested in that study) compared to that of the wild-type strain was also more marked in IFN-γ-activated macrophages than in unactivated ones (11).

A clear relationship between *E. faecalis* virulence and Msr enzymes has been demonstrated in two animal models. Whereas the contribution of Msr deficiencies to virulence in the *Galleria* model was modest, a significant decrease in virulence was evident in a systemic and urinary infection model. Since evidence in support of a role of Msr in bacterial adherence and biofilm formation has been presented (4, 9, 37), one might suggest that defects in these processes in the *msr* mutants could be the basis for the observed reduction in virulence in these models. However, no difference in adherence in comparison to that of the wild-type strain was observed for the *msrA and msrB* mutants using CaCo2 cells as a model (unpublished results). Nevertheless, further experiments on adherence using other cell lines and construction of *msr* mutants in a strong biofilm-forming strain of *E. faecalis* are needed to examine the influence of Msr enzymes on these processes in more detail.

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